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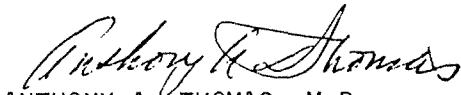
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," DHEW 73-23.

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FOR THE COMMANDER



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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Dog red blood cells were exposed <u>in vivo</u> to phenylhydrazine or monomethyl- hydrazine by injecting the dogs subcutaneously with these compounds. The cell damage was evidenced clinically by hemolytic anemia at all concentrations in the phenylhydrazine exposed animals. In contrast, there was little clinical evidence in monomethylhydrazine cells except at the highest concentration. The freeze- cleave technique showed that phenylhydrazine exposed erythrocytes contained Heinz bodies within two hours. The Heinz bodies formed in the central cytoplasm		

and migrated to the membrane. The membrane was evaginated and dimpled where Heinz bodies were near the membrane and this might represent the damage which initiates sequestration of erythrocytes by the spleen and liver. The freeze-cleave technique showed that monomethylhydrazine treated cells did not produce typical Heinz bodies, but did produce aggregates of granules arranged in ordered rows which may represent hemoglobin crystals.

These striking differences between the effects of phenylhydrazine and monomethylhydrazine exposure may be related to the doses used and route of administration or might indicate widely different toxicities of these compounds.

## PREFACE

This study was initiated in the Pathology Branch, Toxic Hazards Division of the Aerospace Medical Research Laboratory. The author wishes to acknowledge and thank several individuals for their invaluable contributions to this work. Neil S. McNutt, Major, USAF, MC, Chief, Ultrastructural Research Laboratory, for technical comments, assistance and manuscript editing; Ernest E. McConnell, Major, USAF, VC, Chief of the Pathology Branch, for manuscript editing and assistance with the pathology; and Ms. Marilyn George for assistance with the biochemistry.

## INTRODUCTION

The Air Force has been interested in the toxic effects of monomethylhydrazine (MMH) because of its use as a propulsion fuel. Haun,<sup>8</sup> MacEwen,<sup>14</sup> Leahy,<sup>7</sup> and others<sup>12,13</sup> have investigated the acute and chronic effects of MMH exposure, via injection, and inhalation routes, using mice, rats, dogs, monkeys, and in some cases, human volunteers. In all of these studies, the hematologic system was the one most sensitive to damage after exposure to low levels of MMH, with resultant anemia and Heinz body formation. Heinz body formation using phenylhydrazine has been investigated previously by Beaven<sup>1</sup> and by Jandl<sup>3</sup> who demonstrated that the many drugs which induce Heinz body formation are highly reactive redox compounds. These compounds, in the presence of oxygen, cause a progressive oxidative destruction of hemoglobin which precipitates as Heinz bodies.<sup>1,3,15</sup>

Two compounds were used in the present experiment, phenylhydrazine and monomethylhydrazine. Both compounds are highly reactive redox compounds which oxidize in the presence of oxygen to intermediates that damage red cells. These two compounds basically can produce a similar type of damage, but they do so to varying degrees and at different rates. The freeze-cleave technique has been shown to be very useful for the study of erythrocyte damage including Heinz body formation<sup>16,17</sup> and was used in the present study.

Previous studies of erythrocyte damage using dogs exposed to MMH have been done in vivo by inhalation, by intraperitoneal (ip) injection, or by in vitro studies. Erythrocyte damage induced by phenylhydrazine has been studied in vitro using various animal and human cells and in vivo using rabbits.<sup>11</sup> Rifkind used intramuscular (im) injections in rabbits and made significant observations using the standard thin section method.

The purpose of this paper is to present the findings of a study designed to investigate erythrocyte damage to dog red blood cells in vivo induced by subcutaneous (SC) injections of phenylhydrazine and MMH. Most other studies have used either light microscopy, the standard thin sections or, occasionally, freeze-etching. In this study, we used the freeze-etching technique to evaluate erythrocyte damage and attempted to verify Rifkind's results for phenylhydrazine using standard thin sections. We also compared the results of inhalation exposure of MMH to subcutaneous injection in an effort to determine the significance of the route of exposure versus the effect of exposure.

## MATERIALS AND METHODS

### Animals

Eight young adult (18 months old - average weight 9.6 kg) male Beagle dogs (Hazelton Research Animals, Inc, Cumberland, Virginia) were used. Three were exposed to phenylhydrazine, three to monomethylhydrazine (MMH) and two were used as control animals. The agents were administered by subcutaneous (SC) injection on two consecutive days and the animals were sacrificed on the fifth day. The pure phenylhydrazine (Matheson, Coleman, and Bell, Norwood, Ohio) was diluted to a 25% solution by the addition of 0.9% NaCl. Enough hydrochloric acid (1N) was added to reduce the pH to 6.6 in order to obtain a completely miscible solution. Each of the three dogs received a different dose, i.e. 20mg/kg (0.74ml), 30mg/kg (1.0ml), and 40mg/kg (1.4ml). The MMH (Eastman Organic Chemical Company, Rochester, New York) was diluted to a 2% solution with isotonic phosphate-buffered saline, pH 7.4, (containing 0.011mm glucose) in a manner similar to that described by Jacob<sup>21</sup>. The dogs exposed to MMH were injected subcutaneously with either 0.5mg/kg (0.3ml), 1.0mg/kg (0.5ml), or 1.5mg/kg (0.8ml). The control dogs were not injected. Daily blood samples (8ml) were taken from each dog for four days prior to the first injection to serve as baselines, and at two hours post-injection for each of the two exposure days and subsequently at the same time sequence for two additional days.



### Hematology and Chemistry

Baseline values included hematocrit, red cell count, reticulocyte count, white blood cell count with differential, and blood glucose. The hematology was determined using routine clinical laboratory procedures, and the blood glucose was determined using the glucose oxidase method (Worthington Biochemical Corp.). Eight ml of blood was drawn from each dog two hours post injection. Six ml of the sample was heparinized, stored in ice, and centrifuged within twenty minutes for clinical laboratory determinations and freeze-cleaving. The remaining two ml of unheparinized blood was used to determine the methemoglobin and reduced glutathione (GSH) levels. Methemoglobin was determined by a modification of the Evelyn and Malloy method as described by Hainline.<sup>19</sup> The GSH level was measured by the Alloxan 305 method<sup>20</sup> and results reported as  $\mu\text{g GSH}/10^9$  red cells.

### Light Microscopy

Wet preparations of exposed and control cells were stained with 1% crystal violet to determine Heinz body formation. The crystal violet solution was prepared using a saturated solution of crystal violet 2-b stain<sup>18</sup> diluted 1 to 4 with 0.9% sodium chloride. Two drops of cells were mixed with four drops of stain and were incubated at 37°C for 15 minutes. A complete necropsy with routine histopathology was performed on all dogs.

## Electron Microscopy

Blood samples for freeze-etching were prepared by centrifuging the heparinized blood at 4°C for 10 minutes at 2000rpm. The buffy coat was removed and the red cells were washed three times in 0.9% sodium chloride. The cells were suspended in 10, 15, and 20% glycerol for 15 minutes each, and for thirty minutes in 30% glycerol. The glycerol solutions (pH 7.4) were made with isotonic phosphate buffered saline. The red cells were pelleted by centrifuging at 2000rpm for 10 minutes. Samples of the pellets were frozen in Freon 22 at -150°C and then stored in liquid nitrogen until ready for use. The specimens were fractured in vacuo ( $10^{-6}$  Torr) at -115°C by two to three strikes of the microtome blade in a Balzer's 360M Freeze-Etch Machine (Balzer's High Vacuum Co., Santa Ana, California). Replicas of the fractured faces were made by platinum-carbon shadowing according to a technique by Moore.<sup>6</sup> The replica was cleaned with Clorox<sup>®</sup>, washed with distilled water, and mounted on Formvar-coated 300 mesh copper grids. The replicas were examined and photographed in a JEM 100-B electron microscope. (JEOL, USA, Medford, Mass.)

## RESULTS

### Hematology and Chemistry

The hematology results of the animals exposed to phenylhydrazine showed a significant reduction in the hematocrit (Table I), hemoglobin (Table II), red cell counts (Table III), and high methemoglobin levels

TABLE I  
EFFECTS OF PHENYLHYDRAZINE  
ON HEMATOCRIT (VOLUME %)

	<u>DOSE</u>			
	<u>CONTROL</u>	<u>20mg/kg</u>	<u>30mg/kg</u>	<u>40mg/kg</u>
6 JUNE 73	44	46	41	42
7 JUNE 73	42	45	41	44
8 JUNE 73	44	45	41	43
11 JUNE 73	41	47	42	44
12 JUNE 73*	40	45	40	41
13 JUNE 73*	42	33	41	X
14 JUNE 73	42	28	30	X
15 JUNE 73	40	27	24	X

X = dog died

\* = Phenylhydrazine injected

TABLE II  
EFFECTS OF PHENYLHYDRAZINE  
ON HEMOGLOBIN (GRAMS %)

	<u>DOSE</u>			
	<u>CONTROL</u>	<u>20mg/kg</u>	<u>30mg/kg</u>	<u>40mg/kg</u>
6 JUNE 73	14.7	15.0	14.0	14.3
7 JUNE 73	14.3	16.3	14.3	15.0
8 JUNE 73	14.7	15.0	14.0	14.0
11 JUNE 73	14.3	16.3	14.3	15.7
12 JUNE 73*	14.0	16.3	14.0	15.0
13 JUNE 73*	14.3	11.1	14.3	X
14 JUNE 73	14.7	10.5	11.1	X
15 JUNE 73	14.0	8.7	8.5	X

X = dog died

\* = Phenylhydrazine injected

TABLE III  
EFFECTS OF PHENYLHYDRAZINE ON  
RED BLOOD CELL COUNTS (MILLION CELLS)

	<u>DOSE</u>			
	<u>CONTROL</u>	<u>20mg/kg</u>	<u>30mg/kg</u>	<u>40mg/kg</u>
6 JUNE 73	6.09	6.59	6.23	6.07
7 JUNE 73	6.12	6.83	6.20	6.38
8 JUNE 73	5.84	6.32	5.91	6.12
11 JUNE 73	5.84	6.69	6.23	6.44
12 JUNE 73*	5.84	5.77	6.01	5.20
13 JUNE 73*	5.76	4.62	5.59	X
14 JUNE 73	5.72	4.02	4.20	X
15 JUNE 73	5.78	3.35	3.29	X

X = dog died

\* = Phenylhydrazine injected

(Table IV). The dog given 40mg/kg showed 46.9% methemoglobin level two hours post injection on the first day and died 1 1/2 hours after the second injection. The methemoglobin was as high as 85.5% two hours after the last injection in one dog (20mg/kg) and 60.8% in the other (30mg/kg). White blood cell counts, glucose and differentials were within normal limits. Reticulocytes could not be counted because of the presence of numerous Heinz bodies.

The MMH treated animals showed no significant change in hematocrit (Table V) or reticulocyte count (Table VI), but at (1.5mg/kg) there was a slight decrease in hemoglobin (Table VII). The biochemical data did not indicate any change in GSH or production of methemoglobin (Table VIII).

#### Light Microscopy

In each dog exposed to 20, 30, or 40mg/kg phenylhydrazine, 30-40% of the red blood cells contained Heinz bodies at two hours post injection. By 24 hours after the first exposure, Heinz bodies appeared in 95-100% of the cells. The Heinz bodies first appeared as numerous small bodies scattered throughout the cytoplasm; however, after 24 hours they were fewer and larger.

By 24 hours after the second injection of 20, and 30mg/kg phenylhydrazine, the dogs showed hematuria, and nearly 100% of their red cells contained Heinz bodies. The dog given 40mg/kg could not be compared because of death. In contrast, the dogs exposed to MMH at the levels used in this experiment did not demonstrate formation of typical Heinz bodies.

TABLE IV  
EFFECTS OF PHENYLHYDRAZINE  
ON GSH AND METHEMOGLOBIN

			<u>DOSE</u>			
			<u>CONTROL</u>	<u>20mg/kg</u>	<u>30mg/kg</u>	<u>40mg/kg</u>
8	JUNE	73 GSH	53.01	51.38	44.76	45.77
"	"	" MET	0	0	0	0
11	JUNE	73 GSH	49.93	45.64	38.86	38.54
"	"	" MET	0	0	0	0
12	JUNE	73 GSH*	54.60	37.47	39.92	37.69
"	"	" MET*	0	42.9%	0	46.7%
13	JUNE	73 GSH*	50.61	32.08	33.49	X
"	"	" MET*	0	85.5%	60.8%	X
14	JUNE	73 GSH	55.47	37.46	36.18	X
"	"	" MET	0	43.4%	35.9%	X

GSH = Glutathione ( $\mu\text{g. GSH}/10^9\text{ cells}$ )

MET = Methemoglobin (%)

X = dog died

\* = Phenylhydrazine injected

TABLE V  
EFFECTS OF MMH ON  
HEMATOCRIT (VOLUME %)

			<u>DOSE</u>			
			<u>CONTROL</u>	<u>0.5mg/kg</u>	<u>1.0mg/kg</u>	<u>1.5mg/kg</u>
22	Aug.	73	47	47	49	44
23	"	"	45	48	46	42
24	"	"	48	42	48	47
27	"	"	45	46	45	42
28	"	"*	45	45	45	41
29	"	"*	49	46	44	41
30	"	"	45	43	44	41
31	"	"	44	43	41	42

\* = MMH injected



TABLE VI  
EFFECTS OF MMH ON  
RETICULOCYTES (%)

			<u>DOSE</u>			
			<u>CONTROL</u>	<u>0.5mg/kg</u>	<u>1.0mg/kg</u>	<u>1.5mg/kg</u>
22	Aug.	73	1.4	0.6	1.2	1.0
23	"	"	0.9	0.8	0.7	1.4
24	"	"	1.1	1.5	0.8	1.4
27	"	"	0.4	0.4	0.6	0.6
28	"	"*	0.5	0.6	0.6	0.5
29	"	"*	0.4	0.5	0.8	1.2
30	"	"	0.5	0.4	0.9	0.7
31	"	"	0.4	0.4	0.4	0.8

\* = MMH INJECTED

TABLE VII  
EFFECTS OF MMH ON  
HEMOGLOBIN (GRAMS %)

			<u>DOSE</u>			
			<u>CONTROL</u>	<u>0.5mg/kg</u>	<u>1.0mg/kg</u>	<u>1.5mg/kg</u>
22	Aug.	73	16.8	16.8	17.3	15.0
23	"	"	15.0	16.3	15.7	14.4
24	"	"	16.3	15.7	16.3	15.7
27	"	"	15.7	16.3	16.3	14.3
28	"	"*	15.7	15.7	16.3	14.0
29	"	"*	16.3	16.3	14.3	13.7
30	"	"	15.0	14.7	14.7	12.9
31	"	"	16.3	14.8	14.7	13.2

\* = MMH injected

TABLE VIII  
EFFECTS OF MMH ON  
GSH AND METHEMOGLOBIN

			<u>DOSE</u>			
			<u>CONTROL</u>	<u>0.5mg/kg</u>	<u>1.0mg/kg</u>	<u>1.5mg/kg</u>
27 Aug. 73	GSH		57.28	53.04	63.45	45.12
" "	"	MET	0	0	0	0
28 AUG. 73	GSH*		56.64	59.18	66.78	53.66
" "	"	MET*	0	0	0	0
29 AUG. 73	GSH*		56.98	58.08	#	62.35
" "	"	MET*	0	0	#	0
30 AUG. 73	GSH		49.84	51.08	64.27	57.51
" "	"	MET	0	0	0	0

GSH = Glutathione ( $\mu\text{g. GSH}/10^9\text{cells}$ )

MET = Methemoglobin (%)

# = Specimen contaminated

\* = MMH injected

At necropsy the dogs exposed to phenylhydrazine showed a very dark brown color in all visceral organs, and severe congestion in the spleen, liver, and kidney. The spleens were 3 to 5 times normal size. Microscopically, the spleen, liver, and kidney each contained large amounts of blood pigments that did not stain with the Prussian blue iron stain. The Kupffer cells in the liver and the epithelium lining the convoluted tubules of the kidney were hypertrophied and filled with blood pigment (Fig. 1) which was interpreted as hemoglobin based on their appearance and a negative iron stain. Also, there was a striking reduction in spermatogenesis in these exposed animals, and sperm was absent in the epididymis.

Necropsy of the dog injected with 0.5mg/kg MMH showed no macroscopic or microscopic lesions referable to the exposure. At 1.0mg/kg of MMH, bile stasis was suspected in the liver, but blood pigment was not prominent in the Kupffer cells. The kidney showed fine brown granules (probably hemoglobin) in the cells lining the convoluted tubules (Fig. 2). At 1.5mg/kg of MMH, there was increased blood pigment in Kupffer cells and mild fatty changes in the liver. The kidney showed more of the fine brown granular pigment in the cells lining the convoluting tubules, and the spleen showed an increased number of pigment laden macrophages.

#### Electron Microscopy

The freeze-etch technique has been shown to reveal four different surfaces in pure suspensions of red blood cells.<sup>5,10</sup> During the fracture step, the fracture plane passes within the interior of the membrane,

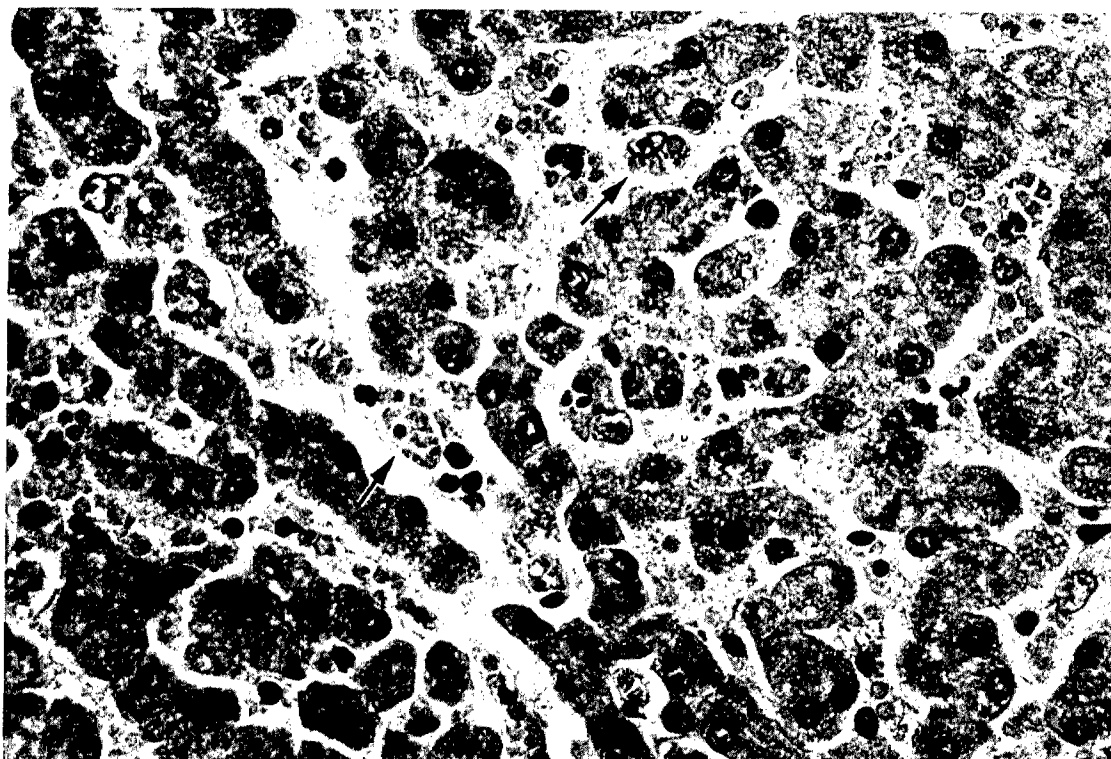


FIGURE 1. Photomicrograph of liver exposed to phenylhydrazine (30mg/kg) 48 hrs. post injection. Kupffer cells (arrow) are enlarged and contain erythrocytes and blood pigment. Hematoxylin and eosin stain. x600.

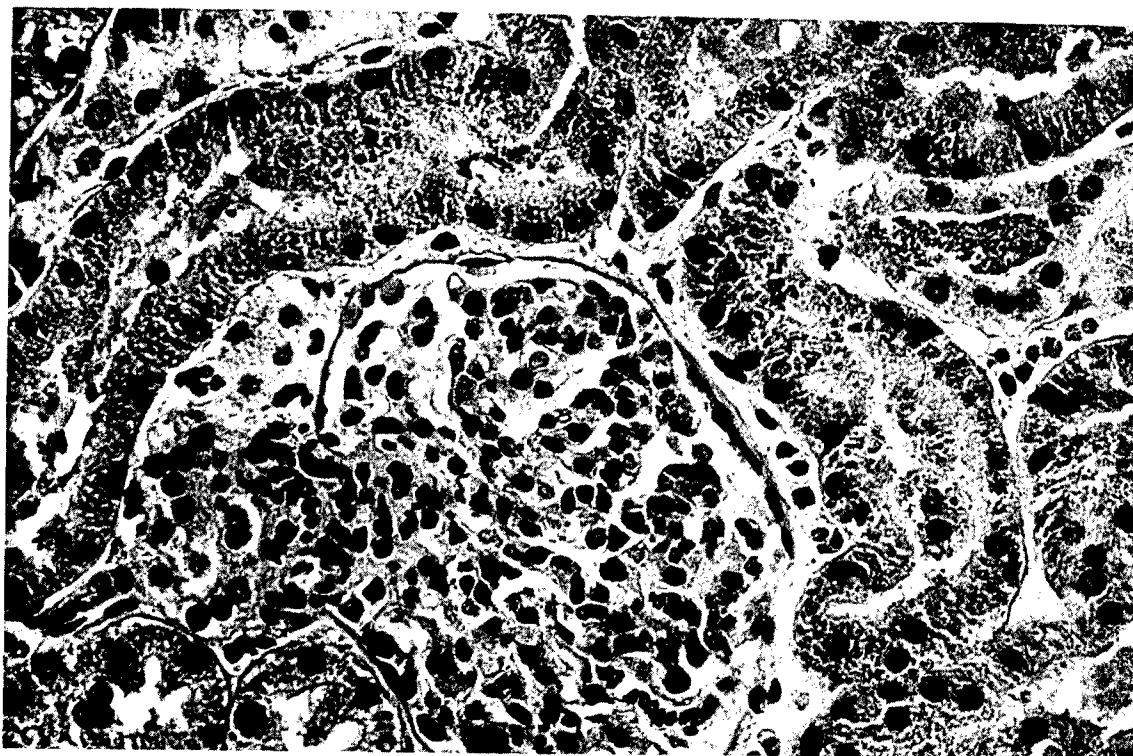


FIGURE 2. Photomicrograph of kidney exposed to phenylhydrazine (30mg/kg) 48 hrs. post injection. Glomerulus surrounded by convoluted tubules which contain fine brown granules (probably hemoglobin) within epithelial lining cells. Hematoxylin and eosin stain. x600.

thus generating two distinct surfaces designated, by convention, as face A and B.<sup>5</sup> The other two surfaces are the fractured cytoplasm and the extracellular space. Face A is the exposed aspect of the inner half membrane and normally has many (60 to 120<sup>0</sup>A) particles randomly distributed over its surface. The cytoplasm has a granular appearance due to molecules of hemoglobin. Face B is the exposed aspect of the outer half membrane and has fewer particles distributed over its surface than face A. The extracellular space usually appears rather smooth in well washed preparations.

The majority of the cells exposed to phenylhydrazine had a normal distribution of particles on the A and B faces of the membranes. There were many small Heinz bodies in the cytoplasm and in some areas appeared to be clustering together to form larger aggregates. Crystalline arrays of granules were present in some of the cells which contained typical Heinz bodies (Fig. 3), and similar crystalline arrays were seen in some control cells. Some of the membranes were distorted and evaginated. Where a Heinz body was adjacent to the membrane surface, the membrane was pulled inward slightly or "dimpled" (Fig. 3 and 4).

The freeze cleaved cells exposed to MMH had A and B faces of the membranes that were relatively undistorted but had evaginations and dimples. The particles were randomly distributed over the A and B faces in a normal manner. Typical Heinz bodies were not seen. Instead, there was a crystalline organization to the hemoglobin into regular parallel rows of granules which in some instances were near evaginations of the membrane. These crystalline structures were numerous and varied in size, but were generally larger than Heinz bodies.

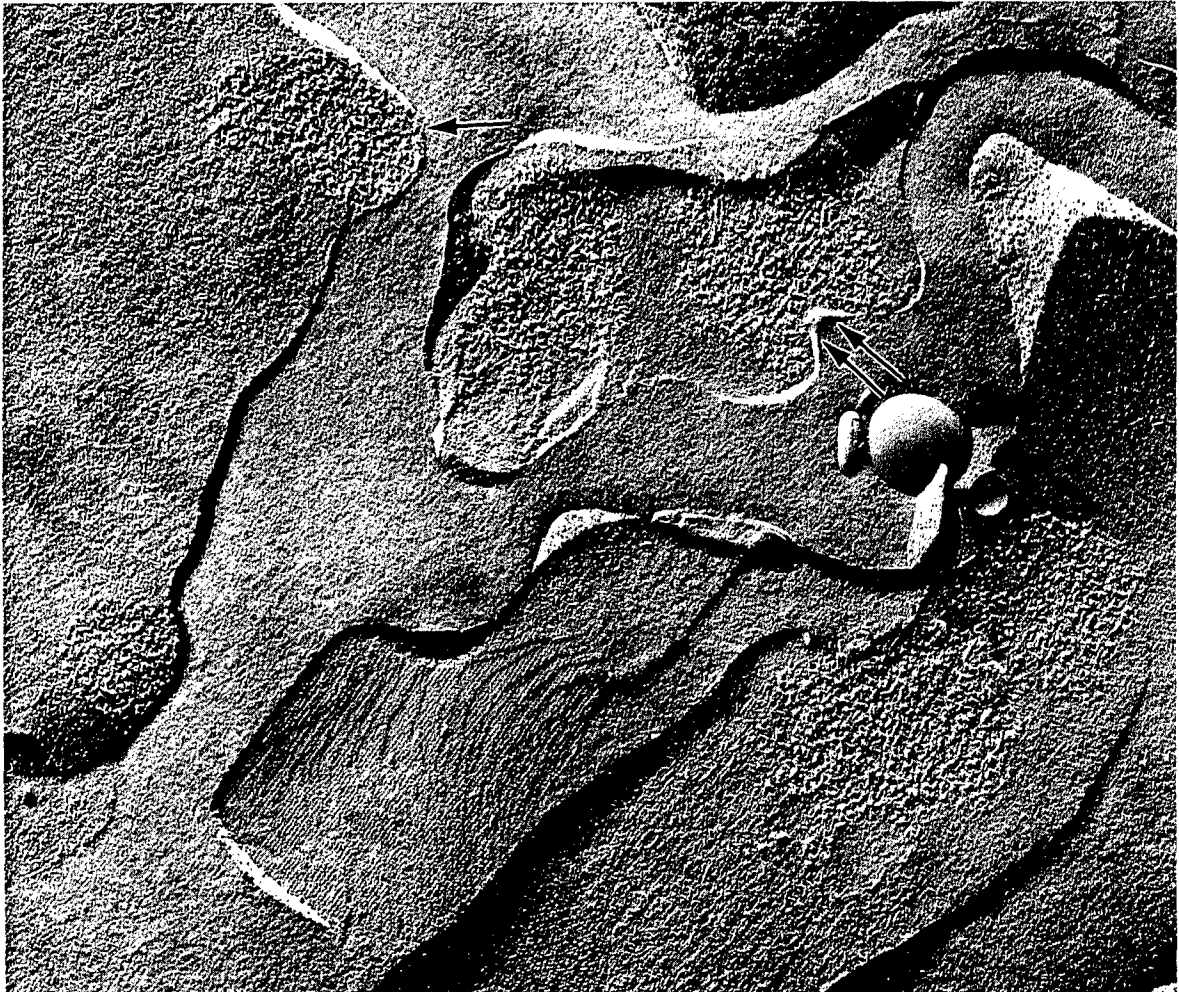


FIGURE 3. Freeze-etched replica of phenylhydrazine treated red cells (30mg/kg) 48 hrs. post injection, showing crystalline arrays of granules. The single arrow shows a distorted membrane evaginated by a Heinz body pushing outward. The double arrow shows a Heinz body adjacent to the membrane and pulling it inward. The direction of shadowing is from bottom to top. x72,000.



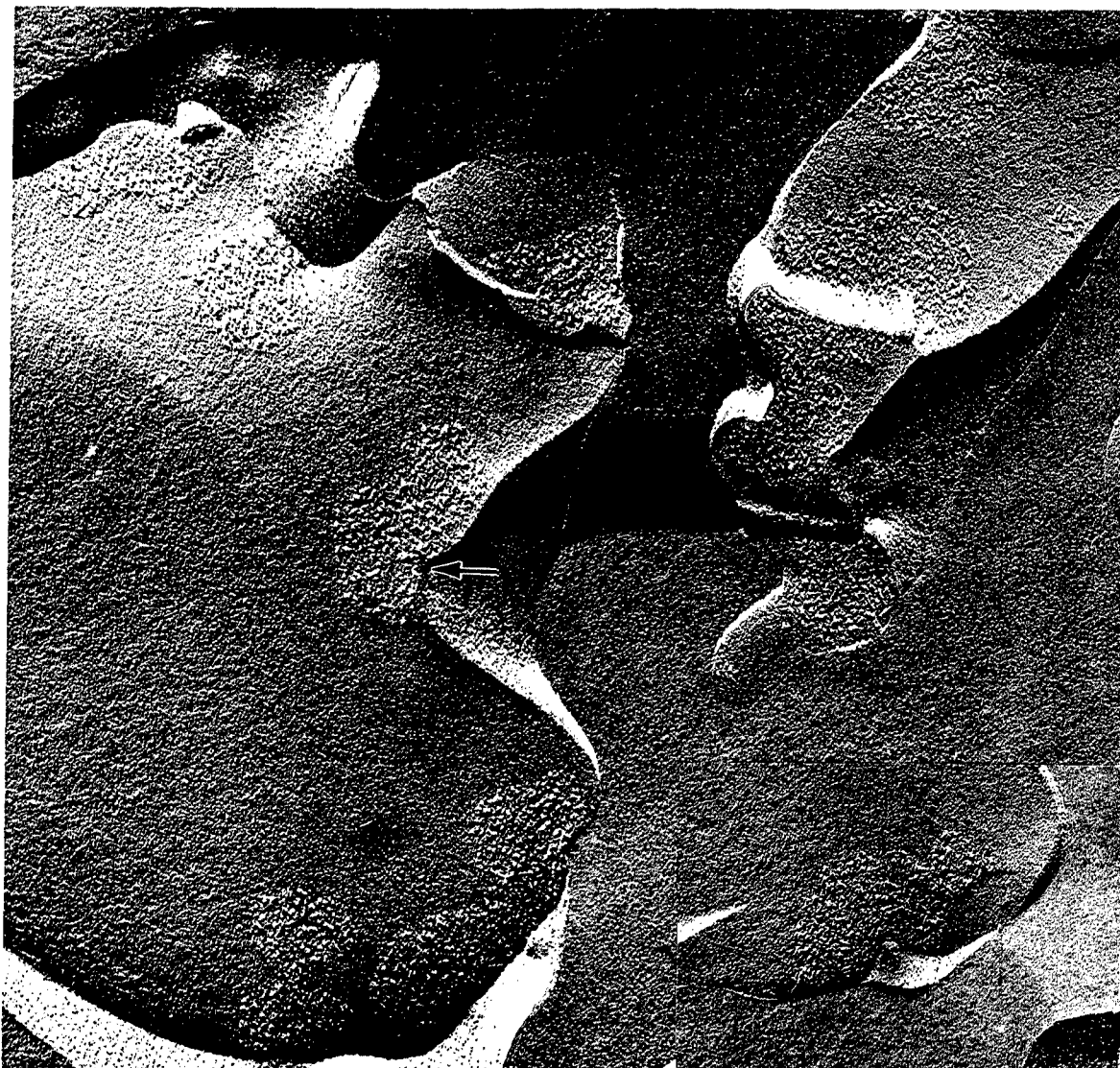


FIGURE 4. Freeze-etched replica of phenylhydrazine treated red cells (20mg/kg) 48 hrs. post-injection. The arrow shows a Heinz body invaginating the inner half membrane (Face A) where they come in contact. x62,000. INSERT: The outer half membrane is pulled in or "dimpled" by a Heinz body. The direction of shadowing is from bottom to top. x60,000.

## DISCUSSION

In the present phenylhydrazine study, hemolytic anemia was observed within the first 24 hours and Heinz Bodies were present in 95-100% of the red cells. The dogs exposed to 20 and 40 mg/kg of phenylhydrazine showed moderate concentrations (42-46%) of methemoglobin the first day, and the 20mg/kg dog showed a methemoglobin level of 85% the second day. The 40mg/kg dog died 1 1/2 hours following the second injection. Twenty-four hours after the last injection, the methemoglobin level was reduced to 43% in the 20mg/kg dog (a 50% reduction), and to 35% in the 30mg/kg dog (a 45% reduction). The 30mg/kg dog showed no methemoglobin the first day but 65% the second day. An explanation for this is not known but may be related to an error in determination the first day.

It has been well documented that various reactive redox compounds produce similar hematological changes in red blood cells. Rifkind and Danon<sup>9</sup> injected rabbits with 15mg/kg of phenylhydrazine and observed a prompt hemolytic anemia and reticulocytosis, while Ham and coworkers<sup>4</sup> injected 10-15mg/kg of acetylphenylhydrazine in dogs and observed hemolytic anemia, reticulocytosis, and reduced GSH within 24 hours. MacEwen<sup>14</sup> found that inhalation exposure of dogs and monkeys to MMH at low concentrations also caused a hemolytic response.

Heinz body formation started in the central cytoplasm and after 48 hours was seen near the cell surface, with the cell membrane being evaginated and dimpled. At the highest concentration of phenylhydrazine (40mg/kg), the Heinz bodies were smaller and were for the most part restricted to

the central cytoplasm. This dog expired at 25 1/2 hours post-injection (1 1/2 hours post-second-injection); therefore, Heinz bodies may not have had sufficient time to migrate to the cell membrane.

There was severe congestion in the spleen, liver, and kidney at each exposure but it was greater in severity at the highest concentration. Histopathological examination of all visceral organs showed evidence of hemolysis and biochemical determinations showed methemoglobin formation in the blood. This indicated that the hemolytic process and oxidative denaturation of hemoglobin had taken place very rapidly. Hemoglobin is oxidized to methemoglobin and the cell was unable to reverse the process at the exposure levels used. A methemoglobin intermediate, an insoluble brown "sulfhemoglobin-like" denatured pigment, appears which precipitates to form Heinz bodies.<sup>3</sup> Heinz body formation mechanism is reversible up to the point of precipitation of pigment from the hemoglobin. Because these erythrocytes have been altered by Heinz body formation they are rapidly removed by the spleen and liver. The abnormal cells are unable to pass through the splenic chords and liver sinusoids thereby causing congestion. As expected, the highest dose level induced the greatest response and caused the most congestion.

It is difficult to understand why the dogs injected with MMH showed no clinical signs of anemia and no indication of a notable change in GSH production or methemoglobin since other investigators<sup>7,8,13,14</sup> using this compound have shown hemolytic changes and Heinz body formation. For

example, Haun<sup>8</sup> exposed dogs to MMH by continuous inhalation exposure and produced Heinz bodies and hemolytic anemia, and Weinstein and George<sup>16</sup> exposed human red cells to MMH in vitro and obtained Heinz bodies and decreased glutathione levels within two hours. Ham and coworkers<sup>4</sup> found similar results in an in vivo study with dogs using acetylphenylhydrazine.

The lack of response in the present study could be related to the low dose of MMH used. A level of 1.5mg/kg was chosen as maximum dose level to insure adequate time for Heinz body formation. This was because an unpublished experiment showed that dogs given 2.5mg/kg MMH daily for 2 days died before Heinz bodies could form (Back and George, verbal communication, August 1973). In addition, the lack of observed response to MMH may be related to reversibility of the response. Decreased GSH level and production of methemoglobin are reversible reactions because of strong protective mechanisms in the cell.<sup>16</sup> It is probable that methemoglobin was produced and GSH levels decreased, but were not detected at the time sequence used for sampling.

The aggregates of granules found arranged in rows within the cytoplasm gave the appearance of an ordered structure, possibly hemoglobin crystals (Fig. 5). Although these crystalline structures were more numerous and larger than typical Heinz bodies, they may be related to an early phase of Heinz body formation similar to that observed by Rifkind and Danon.<sup>9</sup> Their ultrastructural study of Heinz body formation used standard thin sections. They separated Heinz body formation into three stages, an initial, an intermediate, and an end stage. The initial stage was an ordered and possibly crystalline structure which they presumed to be an early phase of hemoglobin oxidation.



FIGURE 5. Freeze-etched replica of monomethylhydrazine (MMH) treated red cells (1.5mg/kg) 48 hrs. post-injection showing the arrangement of granules that give the appearance of an ordered structure, possibly hemoglobin crystals. The direction of shadowing is from bottom to top. x120,000.

Histopathological sections of MMH exposed dogs showed no evidence of Heinz body formation and red cell destruction except at the highest dose. At 0.5mg/kg MMH there was only suspected bile stasis in the liver, and at 1.0mg/kg MMH there were a few fine granules in the cells lining the convoluted tubules. At 1.5mg/kg of MMH there were fatty changes in the liver and hemosiderosis in the spleen, liver, and kidney. These same changes were seen in MMH exposed dogs by Sopher and Robinson.<sup>12</sup> Since the two lower concentrations did not show significant changes, it was felt that the dose levels were at the low end of the response curve.<sup>13,14</sup> The response curve for MMH in dogs is very steep, and to obtain a non-convulsive dose level with a hematologic response would be difficult and would require further study.

In both phenylhydrazine and MMH exposed red blood cells, the distribution of intramembranous particles was not obviously altered in the majority of cells when compared to control preparations. However, the evaginations and dimpling of the membrane when Heinz bodies were adjacent to the membrane may represent a type of red cell damage that causes the red cell to be removed by splenic and hepatic phagocytes.<sup>11</sup> Red cells containing Heinz bodies are removed by sequestration in fixed macrophages. These are easily recognized by their engorgement with Heinz bodies and pigments derived from the breakdown of hemoglobin.

#### CONCLUSIONS

This study has shown that red blood cells in dogs exposed in vivo to phenylhydrazine develop hemolytic anemia, decreased GSH, increased methemoglobin levels and develop Heinz bodies. The phenylhydrazine

induced red cell damage in rabbits by intramuscular injection previously shown by Rifkind with standard thin sections was verified by this study using dogs via subcutaneous injections and the freeze-etch technique. It was apparent that the route of administration did not alter the effect of the phenylhydrazine.

The negative results with MMH exposure will require further investigation of the dose levels and routes of administration in order to obtain an effective non-lethal dose but still a hemolytic response. Also needed is an investigation into the kinetics of red cell turnover in vivo in order to be adequately related to the previous in vivo<sup>8,13,14</sup> and in vitro<sup>16</sup> studies of MMH.

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